| FORM PTO-1390 (REV 11-98) | U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE | ATTORNEY'S DOCKET NUMBER 1498-119 |
|---|---|---|

**TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371**

U.S. APPLICATION NO (If known, see 37 C.F.R. 1.5): 09/763824

| INTERNATIONAL APPLICATION NO. | INTERNATIONAL FILING DATE | PRIORITY DATE CLAIMED |
|---|---|---|
| PCT/GB99/03538 | 26 October 1999 | 28 October 1998 |

TITLE OF INVENTION: **NOVEL ENZYME**

APPLICANT(S) FOR DO/EO/US: **SQUIRRELL et al.**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (U.S.C. 371(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11. To 16. Below concern document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.

12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information. **PTO-1449/ International Search Report**
    ☐ This application is entitled to "Small entity" status. ☐ "Small entity" statement attached.

| U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) | INTERNATIONAL APPLICATION NO. | ATTORNEY'S DOCKET NUMBER |
|---|---|---|
| Unknown 09/763824 | PCT/GB99/03538 | 1498-119 |

17. ☒ The following fees are submitted:

| | CALCULATIONS | PTO USE ONLY |
|---|---|---|
| **BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):** | | |
| -- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... $1000.00 | | |
| -- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... $860.00 | | |
| -- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2) paid to USPTO ..... $710.00 | | |
| -- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... $690.00 | | |
| -- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)..... $100.00 | | |
| **ENTER APPROPRIATE BASIC FEE AMOUNT =** | $ 860.00 | |
| Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)). | $ 0.00 | |

| CLAIMS | NUMBER FILED | | NUMBER EXTRA | | RATE | | |
|---|---|---|---|---|---|---|---|
| Total Claims | 29 | -20 = | 10 | X | $18.00 | $ 180.00 | |
| Independent Claims | 1 | -3 = | 0 | X | $80.00 | 0.00 | |
| MULTIPLE DEPENDENT CLAIM(S) (if applicable) | | | | | $270.00 | $ 0.00 | |
| **TOTAL OF ABOVE CALCULATIONS =** | | | | | | $ 1040.00 | |
| Reduction by ½ for filing by small entity, if applicable. Small entity status must also be asserted. (Note: 37 C.F.R. 1.9, 1.27, 1.28). | | | | | | 0.00 | |
| **SUBTOTAL =** | | | | | | $ 1040.00 | |
| Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)). + | | | | | | 0.00 | |
| **TOTAL NATIONAL FEE =** | | | | | | $ 1040.00 | |
| Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). **$40.00** per property + | | | | | | $ 40.00 | |
| Fee for Petition to Revive Unintentionally Abandoned Application ($1240.00 – Small Entity = $620.00) | | | | | | $ 0.00 | |
| **TOTAL FEES ENCLOSED =** | | | | | | $ 1080.00 | |
| | | | | | | Amount to be: refunded | $ |
| | | | | | | Charged | $ |

a. ☒ A check in the amount of $1080.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.
d. ☐ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

**NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

**SEND ALL CORRESPONDENCE TO:**

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**Arthur R. Crawford**
NAME

**25,327** REGISTRATION NUMBER

**February 27, 2001** Date



502362

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of

SQUIRRELL et al.

Atty. Ref.: 1498-119

Serial No. Unknown

Group:

Filed: February 27, 2001

Examiner:

For: NOVEL ENZYME

* * * * * * * * * * *

February 27, 2001

Assistant Commissioner for Patents
Washington, DC 20231
Sir:

**PRELIMINARY AMENDMENT**

In order to place the above-identified application in better condition for examination, please amend the application as follows:

**IN THE CLAIMS**

Claim 4, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 5, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 6, line 1, change "any one of claims 1 to 4" to --claim 1--.

Claim 7, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 9, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 10, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 11, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 12, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 13, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 14, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 15, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 16, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 17, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 19, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 21, lines 1-2, change "any one of the preceding claims" to --claim 1--.

Please cancel Claim 27 without prejudice or disclaimer.

Claim 28, lines 1-2, change "any one of claims 1 to 20" to --claim 1--.

Claim 29, lines 1-2, change "any one of claims 1 to 20" to --claim 1--.

## REMARKS

The above amendments are made to reduce initial filing fees by removing multiple dependent claims.

Respectfully submitted,

**NIXON & VANDERHYE P.C.**

By: _____

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# Novel Enzyme

The present invention relates to novel proteins, in particular mutant luciferase enzymes having increased thermostability as compared to the corresponding wild type enzyme, to the use of these enzymes in assays and to test kits containing them.

Firefly luciferase catalyses the oxidation of luciferin in the presence of ATP, $Mg^{2+}$ and molecular oxygen with the resultant production of light. This reaction has a quantum yield of about 0.88. The light emitting property has led to its use in a wide variety of luminometric assays where ATP levels are being measured. Examples of such assays include those which are based upon the described in EP-B-680515 and WO 96/02665.

Luciferase is obtainable directly from the bodies of insects, in particular beetles such as fireflies or glow-worms. Particular species from which luciferases have been obtained include the Japanese GENJI or KEIKE fireflies, *Luciola cruciata* and *Luciola lateralis*, the East European firefly *Luciola mingrelica*, the North American firefly *Photinus pyralis* and the glow-worm *Lampyris noctiluca*. Other species from which luciferase can be obtained are listed in Ye et al., Biochimica et Biophysica Acta, 1339 (1997) 39-52. Yet a further species is *Phrixothrix* (railroad-worms), as described by Viviani et al., Biochemistry, 38, (1999) 8271-8279.

However, since many of the genes encoding these enzymes have been cloned and sequenced, they may also be produced using recombinant DNA technology. Recombinant DNA sequences encoding the enzymes are used to transform microorganisms such as *E. coli* which then express the desired enzyme product.

The heat stability of wild and recombinant type luciferases is such that they lose activity quite rapidly when exposed to temperatures in excess of about 30°C, particularly over 35°C. This instability causes problems when the enzyme is used or stored at high ambient temperature, or if the assay is effected

under high temperature reaction conditions, for example in order to increase reaction rate.

Mutant luciferases having increased thermostability are known from EP-A-524448 and WO95/25798. The first of these describes a mutant luciferase having a mutation at position 217 in the Japanese firefly luciferase, in particular by replacing a threonine residue with an isoleucine residue. The latter describes mutant luciferases having over 60% similarity to luciferase from *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* but in which the amino acid residue corresponding to residue 354 of *Photinus pyralis* or 356 of the *Luciola* species is mutated such that it is other than glutamate.

The applicants have found yet further mutants which can bring about increased thermostability and which may complement the mutations already known in the art.

The present invention provides a protein having luciferase activity and at least 60% similarity to luciferase from *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis*, *Hotaria paroula*, *Pyrophorus plagiophthalamus* *Lampyris noctiluca*, *Pyrocoelia nayako*, *Photinus pennsylanvanica* or *Phrixothrix*, wherein in the sequence of the enzyme, at least one of

(a) the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase or to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase;

(b) the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase or to residue 234 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase;

(c) the amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase or to residue 297 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase;

(d) the amino acid residue corresponding to amino acid 14 of the *Photinus pyralis* luciferase or to residue 16 of *Luciola*

*mingrelica*, & residue 17 of *Luciola cruciata* or *Luciola lateralis*;

(e) the amino acid residue corresponding to amino acid 35 of the *Photinus pyralis* luciferase or to residue 37 of *Luciola mingrelica* 38 of *Luciola cruciata* or *Luciola lateralis*;

(f) the amino acid residue corresponding to amino acid residue 105 of the *Photinus pyralis* luciferase or to residue 106 of *Luciola mingrelica*, 107 of *Luciola cruciata* or *Luciola lateralis* or 108 of *Luciola lateralis* gene;

(g) the amino acid residue corresponding to amino acid residue 234 of the *Photinus pyralis* luciferase or to residue 236 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis*;

(h) the amino acid residue corresponding to amino acid residue 420 of the *Photinus pyralis* luciferase or to residue 422 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis*;

(i) the amino acid residue corresponding to amino acid residue 310 of the *Photinus pyralis* luciferase or to residue 312 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis*;

is different to the amino acid which appears in the corresponding wild type sequence and wherein the luciferase enzyme possesses has increased thermostability as compared to an enzyme having the amino acid of the corresponding wild-type luciferase of a particular species at this position.

Preferably, the protein has luciferase activity and at least 60% similarity to luciferase from *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis*, *Hotaria paroula*, *Pyrophorus plagiophthalamus* *Lampyris noctiluca*, *Pyrocoelia nayako*, or *Photinus pennsylanvanica*.

In particular, the protein is a recombinant protein which has luciferase activity and substantially the sequence of a wild-type luciferase, for example of *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis*, *Hotaria paroula*, *Pyrophorus plagiophthalamus (Green-Luc GR)*, *Pyrophorus plagiophthalamus (Yellow-Green Luc YG)*, *Pyrophorus plagiophthalamus (Yellow-Luc YE)*, *Pyrophorus plagiophthalamus*

*(Orange-Luc OR), Lampyris noctiluca, Pyrocelia nayako Photinus pennsylanvanica LY, Photinus pennsylanvanica KW, Photinus pennsylanvanica J19,* or *Phrixothrix green ($Pv_{GR}$) or red ($Ph_{RE}$)* but which may include one or more, for example up to 100 amino acid residues, preferably no more than 50 amino acids and more preferably no more than 30 amino acids, which have been engineered to be different to that of the wild type enzyme.

In particular, bioluminescent enzymes from species that can use the substrate D-luciferin (4,5-dihydro-2-[6-hydroxy-2-benzothiazolyl]-4-thiazole carboxylic acid) to produce light emission may form the basis of the mutant enzymes of the invention.

By way of example, where the protein has substantially the sequence of luciferase of *Photinus pyralis*, in accordance with the invention, at least one of

(a) the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase has been changed to be other than threonine;

(b) the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase has been changed to be other than isoleucine;

(c) the amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase has been changed to be other than phenylalanine;

(d) the amino acid residue corresponding to amino acid 14 of the *Photinus pyralis* luciferase has been changed to be other than phenylalanine;

(e) the amino acid residue corresponding to amino acid 35 of the *Photinus pyralis* luciferase has been changed to be other than leucine;

(f) the amino acid residue corresponding to amino acid residue 105 of the *Photinus pyralis* luciferase has been changed to be other than alanine;

(g) the amino acid residue corresponding to amino acid residue 234 of the *Photinus pyralis* luciferase has been changed to be other than aspartic acid;

(h) the amino acid residue corresponding to amino acid residue 420 of the *Photinus pyralis* luciferase has been changed to be other than serine;

(i) the amino acid residue corresponding to amino acid residue 310 of the *Photinus pyralis* luciferase has been changed to be other than histidine.

Where the protein has substantially the sequence of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* enzyme, in accordance with the invention, at least one of

(a) the amino acid residue corresponding to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than glycine (for *Luciola mingrelica* based sequences) or aparagine (for *Luciola cruciata* or *Luciola lateralis*) based sequences;

(b) the amino acid residue corresponding to residue 234 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than serine;

(c) amino acid residue corresponding to residue 297 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than leucine;

(d) amino acid residue corresponding to amino acid 16 of *Luciola mingrelica*, or to amino acid 17 of *Luciola cruciata* or *Luciola lateralis* is other than phenylalanine;

(e) amino acid residue corresponding to residue 37 of *Luciola mingrelica*, or 38 of *Luciola cruciata* or *Luciola lateralis* is other than lysine;

(f) amino acid residue corresponding to amino acid residue 106 of *Luciola mingrelica*, or to amino acid 107 of *Luciola cruciata* or *Luciola lateralis* or to amino acid 108 of *Luciola lateralis gene* is other than glycine;

(g) amino acid residue corresponding to amino acid residue 236 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* is other than glycine;

(h) amino acid residue corresponding to residue 422 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* is other than threonine;

(i) amino acid residue corresponding to amino acid residue 312 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* is other than threonine (for *Luciola mingrelica* based sequences) or valine (for *Luciola cruciata* or *Luciola lateralis*) based sequences.

The particular substituted amino acids in any case which give rise to enhanced thermostability can be determined by routine methods as illustrated hereinafter. In each case, different substitutions may result in enhanced thermostability. Substitution may be effected by site-directed mutagenesis of DNA encoding native or suitable mutant proteins as would be understood by the skilled person. The invention in this case is associated with the identification of the positions which are associated with thermostability.

In general however, it may be desirable to consider substituting an amino acid of different properties to the wild type amino acid. Thus hydrophilic amino acid residues may, in some cases be preferably substituted with hydrophobic amino acid residues and vice versa. Similarly, acidic amino acid residues may be substituted with basic residues.

For instance, the protein may comprise a protein having luciferase activity and at least 60% similarity to luciferase from *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* enzyme wherein in the sequence of the enzyme, at least one of

(a) the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase and to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is mutated and is other than threonine in the case of *Photinus pyralis* luciferase; or

(b) the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase and to residue 234 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is

mutated and is other than isoleucine in the case of *Photinus pyralis* luciferase; or

(c) amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase and to residue 297 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is mutated and is for example, other than phenylalanine in the case of *Photinus pyralis* luciferase;

and the luciferase enzyme has increased thermostability as compared to the wild-type luciferase.

The sequences of all the various luciferases show that they are highly conserved having a significant degree of similarity between them. This means that corresponding regions among the enzyme sequences are readily determinable by examination of the sequences to detect the most similar regions, although if necessary commercially available software (e.g. "Bestfit" from the University of Wisconsin Genetics Computer Group; see Devereux et al (1984) Nucleic Acid Research 12: 387-395) can be used in order to determine corresponding regions or particular amino acids between the various sequences. Alternatively or additionally, corresponding acids can be determined by reference to L. Ye et al., Biochim. Biophys Acta 1339 (1997) 39-52. The numbering system used in this reference forms the basis of the numbering system used in the present application.

With respect to the possible change of the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase, the polar amino acid threonine is suitably replaced with a non polar amino acid such as alanine, glycine, valine, lecine, isoleucine, proline, phenylalanine, methionine, tryptophan or cysteine. A particularly preferred substitution for the threonine residue corresponding to residue 214 in *Photinus pyralis* is alanine. A more preferred substitution is cysteine. However, different polar residues such as asparagine at this position may also enhance the thermostability of the corresponding enzyme having threonine at this position.

Other amino acids which appear at this position in wild-type luciferase enzymes include glycine (*Luciola mingrelica, Hotaria paroula*), asparagine (*Pyrophorus plagiophthalamus, GR, YC, YE and OR, Luciola cruciata, Luciola lateralis, Lampyris noctiluca, Pyrocelia nayako Photinus pennsylanvanica LY, KW, J19*) and serine (position 211 in *Phrixothrix* luciferase). These may advantageously be substituted with non-polar or different non-polar side chains such as alanine and cysteine.

As regards the possible change of the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase, the nonpolar amino acid isoleucine is suitably replaced with a different non polar amino acid such as alanine, glycine, valine, leucine, proline, phenylalanine, methionine, tryptophan or cysteine. Other amino acids appearing at this position in wild type sequences include serine and asparagine (as well as valine or alanine at corresponding position 229 in Phritothix green and red respectively). Suitably, these polar residues are substituted by non-polar residues such as those outlined above. A particularly preferred substitution for the residue corresponding to residue 232 in *Photinus pyralis* luciferase and to residue 234 of *Luciola mingrelica, Luciola cruciata* or *Luciola lateralis* luciferase is alanine, where this represents a change of amino acid over the wild-type sequence.

Changes of the amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase and to residue 297 of *Luciola mingrelica, Luciola cruciata* or *Luciola lateralis* luciferase, may also affect the thermostability of the protein. (This corresponds to position 292 in *Phrixothix* luciferase.) In general, the amino acid at this position is a non-polar amino acid phenylalanine or leucine. These are suitably changed for different non-polar amino acids. For example, in *Photinus pyralis*, the non-polar amino acid phenylalanine is suitably replaced with a different non polar amino acid, such as alanine, leucine, glycine, valine, isoleucine, proline, methionine, tryptophan or cysteine. A particularly preferred

substitution for the phenylalanine residue corresponding to residue 214 in *Photinus pyralis* luciferase is leucine.

Mutation at the amino acid residue corresponding to amino acid 14 of the *Photinus pyralis* luciferase or to amino acid 16 in Luciola luciferase, (13 in *Phrixothrix* luciferase) is also possible. This amino acid residue (which is usually phenylalanine, but may also be leucine, serine, arginine or in some instances tyrosine) is suitably changed to a different amino acid, in particular to a different nonpolar amino acid such as alanine, valine, leucine, isoleucine, proline, methionine or tryptophan, preferably alanine.

Mutation at the amino acid residue corresponding to amino acid 35 of the *Photinus pyralis* luciferase or to amino acid residue 37 in Luciola mingrelica luciferase (corresponding to amino acid 38 in other *Luciola* spp. And in *Phrixothrix*) may also be effective. This amino acid varies amongst wild type enzymes, which may include leucine (*Photinus pyralis)* but also lysine, histidine, glycine, alanine, glutamine and aspartic acid at this position. Suitably the amino residue at this position is substituted with a non-polar amino acid residue or a different non-polar amino acid such as
such as alanine, valine, phenylalanine, isoleucine, proline, methionine or tryptophan. A preferred amino acid at this position is alanine, where this is different to the wild-type enzyme.

Mutations at the amino acid corresponding to position 14 of the *Photinus pyralis* sequence and/or mutation at the amino acid residue corresponding to amino acid 35 of the *Photinus pyralis* luciferase are preferably not the only mutation in the enzyme. They are suitably accompanied by others of the mutations defined above, in particular those at positions corresponding to positions 214, 395 or 232 of *Photinus pyralis* luciferase.

Changes of the amino acid residue corresponding to residue 105 in *Photinus pyralis* luciferase and to residue 106 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase, (102 in *Phrixothrix*) may also affect the thermostability of the protein. In general, the amino acid at this position is a non-polar amino acid alanine or glycine, or serine in *Phrixothrix*. These are suitably changed for different non-polar amino acids. For example, in *Photinus pyralis*, the non-polar amino acid alanine is suitably replaced with a different non polar amino acid, such as phenylalanine, leucine, glycine, valine, isoleucine, proline, methionine or tryptophan. A particularly preferred substitution for the alanine residue corresponding to residue 105 in *Photinus pyralis* luciferase is valine.

Changes of the amino acid residue corresponding to residue 234 in *Photinus pyralis* luciferase and to residue 236 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase (231 in *Phrixothrix*), may also affect the thermostability of the protein. In general, the amino acid at this position is aspartic acid or glycine and in some cases, glutamine or threonine. These are suitably changed for non-polar or different non-polar amino acids as appropriate. For example, in *Photinus pyralis*, the amino acid residue is aspartic acid is suitably replaced with a non polar amino acid, such as alanine, leucine, glycine, valine, isoleucine, proline, methionine or tryptophan. A particularly preferred substitution for the phenylalanine residue corresponding to residue 234 in *Photinus pyralis* luciferase is glycine. Where a non-polar amino acid residue such as glycine is present at this position (for example in *Luciola* luciferase), this may be substituted with a different non-polar amino acid.

Changes of the amino acid residue corresponding to residue 420 in *Photinus pyralis* luciferase and to residue 422 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase (417 in *Phrixothrix* green and 418 in *Phrixothrix* red), may also affect the thermostability of the protein. In general, the

amino acid at this position is an uncharged polar amino acid serine or threonine or glycine. These are suitably changed for different uncharged polar amino acids. For example, in *Photinus pyralis*, the serine may be replaced with asparagine, glutamine, threonine or tyrosine, and in particular threonine.

Changes of the amino acid residue corresponding to residue 310 in *Photinus pyralis* luciferase and to residue 312 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase, may also affect the thermostability of the protein. The amino acid residue at this position varies amongst the known luciferase proteins, being histidine in *Photinus pyralis*, *Pyrocelia nayako*, *Lampyris noctiluca* and some forms of *Photinus pennsylanvanica* luciferase, threonine in *Luciola mingrelica*, *Hotaria paroula* and *Phrixothix* (where it is amino acid 307) luciferase, valine in *Luciola cruciata* and *Luciola lateralis*, and asparagine in some *Pyrophorus plagiophthalamus* luciferase. Thus, in general, the amino acid at this position is hydrophilic amino acid which may be changed for a different amino acid residue which increases thermostability of the enzyme. A particularly preferred substitution for the histidine residue corresponding to residue 310 in *Photinus pyralis* luciferase is arginine.

Other mutations may also be present in the enzyme. For example, in a preferred embodiment, the protein also has the amino acid at position corresponding to amino acid 354 of the *Photinus pyralis* luciferase (356 in Luciola luciferase and 351 in *Phrixothrix*) changed from glutamate, in particular to an amino acid other than glycine, proline or aspartic acid. Suitably, the amino acid at this position is tryptophan, valine, leucine, isoleucine are asparagine, but most preferably is lysine or arginine. This mutation is described in WO 95/25798.

In an alternative preferred embodiment, the protein also has the amino acid at the position corresponding to amino acid 217

in Luciola luciferase (215 in *Photinus pyralis*) changed to a hydrophobic amino acid in particular to isoleucine, leucine or valine as described in EP-A-052448.

The proteins may contain further mutations in the sequence provided the luciferase activity of the protein is not unduly compromised. The mutations suitably enhance the properties of the enzyme or better suit it for the intended purpose in some way. This may mean that they result in enhanced thermostability and/or colour shift properties, and/or the $K_m$ for ATP of the enzymes. Examples of mutations which give rise to colour shifts are described in WO95/18853. Mutations which affect $K_m$ values are described for example in WO 96/22376 and International Patent Application No. PCT/GB98/01026 which are incorporated herein by reference.

Proteins of the invention suitably have more than one such mutation, and preferably all three of the mutations described above.

Proteins of the invention include both wild-type and recombinant luciferase enzymes. They have at least 60% similarity to the sequences of *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* or other luciferase enzymes as discussed above in the sense that at least 60% of the amino acids present in the wild-type enzymes are present in the proteins of the invention. Such proteins can have a greater degree of similarity, in particular at least 70%, more preferably at least 80% and most preferably at least 90% to the wild-type enzymes listed above. Similar proteins of this type include allelic variants, proteins from other insect species as well as recombinantly produced enzymes.

They may be identified for example, in that they are encoded by nucleic acids which hybridise with sequences which encode wild-type enzymes under stringent hybridisation conditions, preferably high stringency conditions. Such conditions would be well understood by the person skilled in the art, and are

exemplified for example in Sambrook et al. (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press). In general terms, low stringency conditions can be defined as 3 x SCC at about ambient temperature to about 65°C, and high stringency conditions as 0.1 x SSC at about 65°C. SSC is the name of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3 x SSC is three times as strong as SSC and so on.

In particular, the similarity of a particular sequence to the sequences of the invention may be assessed using the multiple alignment method described by Lipman and Pearson, (Lipman, D.J. & Pearson, W.R. (1985) Rapid and Sensitive Protein Similarity Searches, Science, vol 227, pp1435-1441). The "optimised" percentage score should be calculated with the following parameters for the Lipman-Pearson algorithm:ktup =1, gap penalty =4 and gap penalty length =12. The sequence for which similarity is to be assessed should be used as the "test sequence" which means that the base sequence for the comparison, such as the sequence of *Photinus pyralis* or any of the other sequences listed above,as recorded in Ye *et al.*, *supra.*, or in the case of *Phrixotrix*, as described in Biochemistry, 1999, 38, 8271-8279, should be entered first into the algorithm. Generally, *Photinus pyralis* will be used as the reference sequence.

Particular examples of proteins of the invention are wild-type luciferase sequence with the mutations as outlined above. The proteins have at least one and preferably more than one such mutation.

The invention further provides nucleic acids which encode the luciferases as described above. Suitably, the nucleic acids are based upon wild-type sequences which are well known in the art. Suitable mutation to effect the desired mutation in the amino acid sequence would be readily apparent, based upon a knowledge of the genetic code.

The nucleic acids of the invention are suitably incorporated into an expression vector such as a plasmid under the control of control elements such as promoters, enhancers, terminators etc. These vectors can then be used to transform a host cell, for example a prokaryotic or eukaryotic cell such as a plant or animal cell, but in particular a prokaryotic cell such as *E. coli* so that the cell expresses the desired luciferase enzyme. Culture of the thus transformed cells using conditions which are well known in the art will result in the production of the luciferase enzyme which can then be separated from the culture medium. Where the cells are plant or animal cells, plants or animals may be propagated from said cells. The protein may then be extracted from the plants, or in the case of transgenic animals, the proteins may be recovered from milk. Vectors, transformed cells, transgenic plants and animals and methods of producing enzyme by culturing these cells all form further aspects of the invention.

The *Photinus pyralis* T214A mutant luciferase was created by random mutagenesis as described hereinafter. It was found that the T214A single point mutation has greater thermostability than wild type luciferase.

Two new triple mutant luciferases: E354K/T214A/A215L and E354K/T214A/I232A were also prepared and these also have exhibited greater thermostability.

Particular examples of mutant enzymes of *Photinus pyralis* which fall within the scope of the invention include the following:

I232A/E354K
T214A/I232A/E354K
A215L/I232A/E354K
T214A/I232A/E354K/A215L
I232A/E354K/T214A/F295L
I232A/E354K/T214A F295L/F14A/L35A
I232A/E354K/T214A/F295L/F14A/L35A/A215L
A105V
T214A

T214C
T214N
T295L
I232A
F14A
L35A
D234G
S420T
H310R

or equivalents of any of these when derived from the luciferases of other species.

The mutations for the creation of the triple mutant were introduced to the luciferase gene on plasmid pET23 by site-directed mutagenesis, (PCR). The oligonucleotides added to the PCR reaction in order to effect the relevant mutations are given in the Examples below.

It has been reported previously that the effect of point mutations at the 354 and 215 positions are additive. This invention provides the possibility of combining three or more such mutations to provide still greater thermostability.

Thermostable luciferase of the invention will advantageously be employed in any bioluminescent assay which utilises the luciferase/luciferin reaction as a signalling means. There are many such assays known in the literature. The proteins may therefore be included in kits prepared with a view to performing such assays, optionally with luciferin and any other reagents required to perform the particular assay.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 illustrates the plasmids used in the production of mutants in accordance with the invention;

Figure 2 shows the results of heat inactivation studies on luciferases including luciferases of the invention;

Figure 3 shows the results of thermostability experiments on various luciferase mutants;

Figure 4 shows the results of thermostability experiments on other luciferase mutants; and

Figure 5 shows oligonucleotides used in the preparation of mutant enzymes of the invention.

Example 1

Identification of Thermostable Mutant Luciferase

The error-prone PCR was based on the protocol devised by Fromant *et al.*, Analytical Biochemistry, 224, 347-353 (1995).

The dNTP mix in this reaction was:
35mM dTTP
12.5mM dGTP
22.5mM dCTP
14mM dATP

The PCR conditions were:

0.5 µl (50ng) plasmid pPW601a J54*

5.0 µl 10x KCl reaction buffer

1 µl each of W56 and W57* (60 pmoles of each primer)

1 µl Biotaq ™ polymerase (5U)

2 µl dNTPs (see above)

1.76 µl $MgCl_2$ (50 mM stock)

1 µl $mNC1_2$ (25mM stock) [final concentration in reaction = 3.26mM]

36.7 µl $dH_20$

*Plasmid pPW601aJ54 is a mutated version of pPW601a (WO 95/25798) where an NdeI site has been created within the 3

bases prior to the ATG start codon. This allows for easy cloning from pPW601a into the pET23 vector.

+Primer sequences:

W56:

5′ – AAACAGGGACCCATATGGAAGACGC – 3′

W57:

5′ – AATTAACTCGAGGAATTTCGTCATCGCTGAATACAG – 3′)

Cycling parameters were:

94°C-5 min

Then 12 x cycles of: 94°C-30s

55°C-30s

72°C-5min

72°C-10 min

The PCR products were purified from the reaction mix using a Clontech Advantage ™ PCR-pure kit. An aliquot of the purified products was then digested with the restriction enzymes NdeI and XhoI. The digested PCR products were then "cleaned up" with the Advantage kit and ligated into the vector pET23a which had been digested with the same enzymes.

Ligation conditions:

4µl pET23a (56ng)

5µl PCR products (200ng)

3µl 5x Gibco BRL ligase reaction buffer

1µl Gibco BRL ligase (10U)

2µl $dH_2O$

The ligation was carried out overnight at 16°C.

The ligated DNAs were then purified using the Advantage™ kit and then electroporated into electrocompetent *E. coli* HB101 cells (1mm cuvettes, 1.8 Kv).

Eleven electroporations were performed and the cells were then added to 40 ml of TY broth containing 50μg/ml ampicillin. The cells were then grown overnight at 37°C. The entire 50ml of culture grown overnight was used to purify plasmid DNA. This is the library.

Screening the library

An aliquot of the plasmid library was used to electroporate *E. coli* BL21 DE3 cells. These cells were then plated onto LB agar containing 50μg/ml ampicillin and grown overnight at 37°C.

The next day, colonies were picked and patched onto nylon filters on LB agar + amp plates and growth continued overnight at 37°C. The next day, filters were overlaid with a solution of luciferin - 500μM in 100mM sodium citrate pH5.0. The patches were then viewed in a darkroom. One colony/patch was picked from 200 for further analysis.

Characterisation of the thermostable mutant

The *E. coli* clone harbouring the mutant plasmid was isolated. Plasmid DNA was prepared for ABI sequencing. The entire open reading frame encoding luciferase was sequenced using 4 different oligonucleotide primers. Sequencing revealed a single point mutation at nt 640 (A → G). Giving a codon change of ACT (T) to GCT (A) at amino acid position 214.

Example 2

Preparation of Triple Mutant Enzyme

A mutagenic oligonucleotide was then used to create this same mutation in pMOD1 (A215L/E354K) to create a triple mutant pMOD2 (A215L/E354K/T214A). This mutation also creates a unique SacI/SstI site in pMOD1.

Example 3

Preparation of further triple mutant enzyme

The following primers were used to create the triple mutant T214A/I232A/E354K using a standard PCR reaction and with the pET23 plasmid with the T214A mutation as template:

CTGATTACACCCAAGGGGGATG E354K-sense
CATCCCCCTTGGGTGTAATCAG E354K-antisense

GCAATCAAATCGCTCCGGATACTGC I232A-sense
GCAGTATCCGGAGCGATTTGATTGC I232A-antisense.

Example 4

Identification of thermostable 295 mutant

The F295 mutant was created using the error-prone PCR method described by Fromant et al., Analytical Biochemistry, vol 224, 347-353 (1995). The PCR conditions used were as follows:

0.5 µl (50 ng) plasmid pET23
5.0 µl 10x KCl reaction buffer
1 µl primer 1 - 60 pmoles of each primer
1 µl primer 2
1 µl Biotaq™ polymerase (5U)
2 µl dNTPs, in mixture 35 mM dTTP, 12.5 mM dGTP, 22.5 mM dCTP, 14 mM dATP
1.76 µl $MgCl_2$ (50 mM stock)
1 µl $MnCl_2$ (25 mM stock) [final concentration in reaction = 3.26 mM]
36.7 µl $dH_2O$
Primer 1 = 5' - AAACAGGGACCCATATGGAAGACGC - 3'
Primer 2 = 5' - AATTAACTCGAGGAATTTCGTCATCGCTGAATACAG - 3'

The cycling parameters were:
94°C for 5 min
15 cycles of: 30 s @ 94°C
30 s @ 55°C
5 min @ 72°C
then 10 min at 72°C

The PCR products were purified from the reaction mix using a Clontech Advantage™ PCR-Pure kit. An aliquot of the purified products was then digested with the restriction enzymes NdeI and XhoI. The digested PCR products were then "cleaned up" with the Advantage™ kit and ligated into the vector pET23a, which had been digested with the same enzymes.

The ligation conditions were as follows:

56 ng pET23a
200 ng PCR products
3 µl 5x Gibco BRL ligase reaction buffer
1µl Gibco BRL ligase (10U)
volume made up to 10 µl with $dH_2O$

The ligation was carried out overnight at 16°C.

The ligated DNAs were then purified using the Advantage™ kit and then electroporated into electrocompetent *Escherichia coli* DH5α cells (1mm cuvettes, 1.8kV). 1ml of SOC broth was added to each electroporation and the cells allowed to recover and express antibiotic resistance genes encoded by the plasmid. Aliquots of the library were inoculated onto LB agar containing 50 µg/ml ampicillin and the bacteria were grown overnight at 37°C. Nylon filter discs were then overlaid onto the agar plates and the colonies transferred to fresh plates. The original plates were left at room temperature for the colonies to re-grow. The plates with the nylon filters were incubated at 42°C for 2 h before plates were sprayed with 500µM luciferin in 100mM citrate buffer pH5.0 and viewed in a darkroom.

Three thermostable colonies were selected on the basis that they still glowed after 2 h at 42°C. Plasmid DNA was isolated from these clones and sequenced, and this revealed the F295L mutation in each case.

Example 5

Other mutants of the invention were produced by PCR using appropriate combinations of the oligonucleotides listed above as well as the following:

GAAAGGCCCGGCACCAGCCTATCCTCTAGAGG F14A-sense
CCTCTAGCGGATAGGCTGGTGCCGGGCCTTTC F14A-antisense

GAGATACGCCGCGGTTCCTGG L35A-sense
CCAGGAACCGCGGCGTATCTC L35A-antisense

Example 6

Purification of luciferase and heat inactivation studies.

Cells expressing the recombinant mutant luciferases were cultured, disrupted and extracted as described in WO 95/25798 to yield cell free extracts of luciferase.

Eppendorf tubes containing the cell free extracts were incubated generally at 40°C unless otherwise stated. Purified preparations of wild type luciferases (for comparative purposes were incubated in thermostability buffer comprising 50mM potassium phosphate buffer pH7.8 containing 10% saturated ammonium sulphate, 1mM dithiothreitol and 0.2% bovine serum albumin (BSA). At set times a tube was removed and cooled in an ice/water bath prior to assay with remaining assayed activity being calculated as a percentage of the initial activity or relative bioluminesce.

The results are illustrated in Figures 2 and 3 hereinafter. It can be seen from Figure 2 that luciferase mutants of the invention have improved thermostability compared with the previously known mutants.

The dramatic increase in stability over wild-type luciferase (RWT) is clear from Figure 3.

Example 7

Investigations into the activity of 214 mutants

A library of 214 mutants was prepared using site-directed mutagenesis using cassette oligos (Figure 5) and thermostable mutants selected and tested as described in Example 1. Three particularly thermostable mutants were characterised by sequencing as described in Example 1 as T214A, T214C and T214N.

O/N cultures of E. coli XL1-Blue harbouring plasmids encoding T214, T214A, T214C and T214N were lysed using the Promega lysis buffer. 50µl of liquid extracts were then heat inactivated at 37°C and 40°C over various time points. Aliquots 10µl of heated extract were then tested in the Promega live assay buffer (100µl).

The results are shown in the following Tables

| | 0 | 4 min | 8 min | 22 min | (37°C) |
|---|---|---|---|---|---|
| rwt T214 | 11074 | 5561 | 2555 | 343 | RLU |
| T214C | 106449 | 92471 | 90515 | 78816 | RLU |
| T214A | 63829 | 52017 | 45864 | 35889 | RLU |
| T214N | 60679 | 49144 | 41736 | 29488 | RLU |

| | | % remaining activity 37°C | | |
|---|---|---|---|---|
| rwt T214 | 100 | 50.2 | 23.1 | 3.1 |
| T214C | 100 | 86.9 | 85.0 | 74.0 |
| T214A | 100 | 81.5 | 71.8 | 56.2 |
| T214N | 100 | 81.0 | 68.8 | 48.6 |

The experiment was repeated at 40°C with the 3 mutants

| | 0 | 4 min | 8 min | 16 min | |
|---|---|---|---|---|---|
| T214C | 104830 | 79365 | 72088 | 56863 | RLU |
| T214A | 64187 | 43521 | 28691 | 14547 | RLU |
| T214N | 60938 | 38359 | 25100 | 12835 | RLU |

| | | % remaining activity 40°C | | |
|---|---|---|---|---|
| | 0 | 4 min | 8 min | 16 min |
| T214C | 100 | 73.7 | 68.8 | 54.2 |
| T214A | 100 | 67.8 | 44.7 | 22.7 |
| T214N | 100 | 63.0 | 41.2 | 21.1 |

These results indicate that T214C is significantly more thermostable than either r-wt or T214A or N. This change in properties is unexpected as it is usually expected that the more cysteine residues that are present, the worse the thermostability.

Example 8

Investigation of other point mutations

A series of other Photinus pyralis mutants with single point mutations were prepared using random error-prone PCR (Figure 5). Following, screening and sequencing of the mutants generated, the sequencing was checked using site-directed mutagenesis followed by further sequencing. These were D234G, A105V and F295L. The thermostability of these mutants as well as recombinant wild-type Photinus pyralis luciferase was tested. Protein samples in Promega lysis buffer were incubated at 37°C for 10 minutes and their activity assayed after 2, 5 and 10 minutes. The results, showing that each mutation produced enhanced thermostability over wild type, is shown in Figure 4.

Claims

1. A protein having luciferase activity and at least 60% similarity to luciferase from *Photinus pyralis, Luciola mingrelica, Luciola cruciata, Luciola lateralis, Hotaria paroula,* Pyrophorus *plagiophthalamus, Lampyris noctiluca, Pyrocoelia nayako or Photinus pennsylanvanica,* wherein in the sequence of the enzyme, at least one of

(a) the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase or to residue 216 of *Luciola mingrelica, Luciola cruciata* or *Luciola lateralis* luciferase;

(b) the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase or to residue 234 of *Luciola mingrelica, Luciola cruciata* or *Luciola lateralis* luciferase;

(c) amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase or to residue 297 of *Luciola mingrelica, Luciola cruciata* or *Luciola lateralis* luciferase;

(d) amino acid residue corresponding to amino acid 14 of the *Photinus pyralis* luciferase or to residue 16 of *Luciola mingrelica,* or 17 of *Luciola cruciata* or *Luciola lateralis;*

(e) amino acid residue corresponding to amino acid 35 of the *Photinus pyralis* luciferase or to residue 37 of *Luciola mingrelica,* or 38 of *Luciola cruciata* or *Luciola lateralis;*

(f) the amino acid residue corresponding to amino acid residue 105 of the *Photinus pyralis* luciferase or to residue 106 of *Luciola mingrelica,* 107 of *Luciola cruciata* or *Luciola lateralis* or 108 of *Luciola lateralis* gene;

(g) amino acid residue corresponding to amino acid residue 234 of the *Photinus pyralis* luciferase or to residue 236 of *Luciola mingrelica, Luciola cruciata* or *Luciola lateralis;*

(h) amino acid residue corresponding to amino acid residue 420 of the *Photinus pyralis* luciferase or to residue 422 of *Luciola mingrelica, Luciola cruciata* or *Luciola lateralis;*

(i) amino acid residue corresponding to amino acid residue 310 of the *Photinus pyralis* luciferase or to residue 312 of *Luciola mingrelica, Luciola cruciata* or *Luciola lateralis;*

is different to the amino acid which appears in the corresponding wild type sequence and wherein the luciferase enzyme has increased thermostability as compared to an enzyme having the amino acid of the corresponding wild-type luciferase at this position.

2. A protein according to claim 1 which has the sequence of a wild-type luciferase, in which more than one amino acid residue is different to that of the wild type enzyme.

3. A protein according to claim 2 wherein up to 50 amino acids are different to that of the wild type enzyme.

4. A protein according to any one of the preceding claims wherein the luciferase is a modified form of luciferase of *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase.

5. A protein according to any one of the preceding claims wherein the sequence of luciferase of *Photinus pyralis*, wherein at least one of

(a) the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase is other than threonine;

(b) the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase is other than isoleucine;

(c) amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase is other than phenylalanine;

(d) amino acid residue corresponding to amino acid 14 of the *Photinus pyralis* luciferase is other than phenylalanine;

(e) amino acid residue corresponding to amino acid 35 of the *Photinus pyralis* luciferase is other than leucine;

(f) amino acid residue corresponding to amino acid residue 105 of the *Photinus pyralis* luciferase is other than alanine;

(g) amino acid residue corresponding to amino acid residue 234 of the *Photinus pyralis* luciferase is other than aspartic acid;

(h) amino acid residue corresponding to amino acid residue 420 of the *Photinus pyralis* luciferase is other than serine;

(i) amino acid residue corresponding to amino acid residue 310 of the *Photinus pyralis* luciferase is other than histidine.

6. A protein according to any one of claims 1 to 4 wherein protein has substantially the sequence of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* enzyme, and wherein at least one of

(a) the amino acid residue corresponding to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than glycine (for *Luciola mingrelica* based sequences) or aparagine (for *Luciola cruciata* or *Luciola lateralis*) based sequences;

(b) the amino acid residue corresponding to residue 234 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than serine;

(c) amino acid residue corresponding to residue 297 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is other than leucine;

(d) amino acid residue corresponding to amino acid 16 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* is other than phenylalanine;

(e) amino acid residue corresponding to residue 37 of *Luciola mingrelica*, or residue 38 of *Luciola cruciata* and *Luciola lateralis* is other than lysine;

(f) amino acid residue corresponding to amino acid residue 106 of *Luciola mingrelica*, 107 of *Luciola cruciata* or *Luciola lateralis*, or 108 of *Luciola lateralis gene* is other than glycine;

(g) amino acid residue corresponding to amino acid residue 236 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* is other than glycine;

(h) amino acid residue corresponding to residue 422 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* is other than threonine;

(i) amino acid residue corresponding to amino acid residue 312 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* is other than threonine (for *Luciola mingrelica* based sequences)

or valine (for *Luciola cruciata* or *Luciola lateralis*) based sequences.

7. A protein according to any one of the preceding claims wherein comprising a protein having luciferase activity and at least 60% similarity to luciferase from *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* enzyme wherein in the sequence of the enzyme, at least one of (a) the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase and to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is mutated and is other than threonine in the case of *Photinus pyralis* luciferase; or
(b) the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase and to residue 234 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is mutated and is other than isoleucine in the case of *Photinus pyralis* luciferase; or
(c) amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase and to residue 297 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is mutated and is for example, other than phenylalanine in the case of *Photinus pyralis* luciferase;
and the luciferase enzyme has increased thermostability as compared to the wild-type luciferase.

8. A protein according to claim 1 wherein the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase and to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is alanine.

9. A protein according to any one of the preceding claims wherein the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase and to residue 234 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is alanine.

10. A protein according to any one of the preceding claims which is a mutated *Photinus pyralis* luciferase wherein the amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase is leucine.

11. A protein according to any one of the preceding claims wherein the amino acid residue corresponding to amino acid 14 of the *Photinus pyralis* luciferase or to amino acid 16 in Luciola luciferase, is alanine.

12. A protein according to any one of the preceding claims wherein the luciferase is a mutated luciferase of *Photinus pyralis* or a *Luciola* species where the amino acid residue corresponding to amino acid 35 of the *Photinus pyralis* luciferase or to amino acid residue 37 in *Luciola mingrelica* or 38 of *Luciola lateralis* or *cruciata* luciferase is alanine.

13. A protein according to any one of the preceding claims wherein the amino acid residue corresponding to residue 105 in *Photinus pyralis* luciferase and to residue 106 of *Luciola mingrelica*, 107 of *Luciola cruciata* or *Luciola lateralis* or 108 of *Luciola lateralis gene* luciferase is valine.

14. A protein according to any one of the preceding claims which comprises a mutated *Photinus pyralis* luciferase wherein the amino acid residue corresponding to residue 234 in *Photinus pyralis* luciferase is glycine.

15. A protein according to any one of the preceding claims which comprises a mutated *Photinus pyralis* luciferase wherein the amino acid residue corresponding to residue 420 in *Photinus pyralis* luciferase is threonine.

16. A protein according to any one of the preceding claims which comprises a mutated *Photinus pyralis* luciferase wherein the amino acid residue corresponding to residue 310 in *Photinus pyralis* luciferase is arginine.

17. A protein according to any one of the preceding claims wherein the amino acid at position corresponding to amino acid 354 of the *Photinus pyralis* luciferase (356 in Luciola luciferase) is other than glutamate.

18. A protein according to claim 17 wherein the amino acid at position corresponding to amino acid 354 of the *Photinus pyralis* luciferase (356 in Luciola luciferase) is lysine or arginine.

19. A protein according to any one of the preceding claims wherein the amino acid at the position corresponding to amino acid 217 in Luciola luciferase (215 in *Photinus pyralis*) is a different hydrophobic amino acid.

20. A protein according to claim 19 wherein the amino acid at the position corresponding to amino acid 217 in Luciola luciferase (215 in *Photinus pyralis*) is isoleucine, leucine or valine.

21. A nucleic acid which encodes a luciferase according to any one of the preceding claims.

22. A vector comprising a nucleic acid according to claim 21.

23. A cell transformed with a vector according to claim 22.

24. A cell according to claim 23 which is a prokaryotic cell.

25. A cell according to claim 23 which is a plant cell.

26. A plant comprising cells according to claim 25.

27. A method of producing a protein according to any one of claims 1 to 20, which method comprises culture of a cell according to claim 23 or growth of a plant according to claim 26.

28. The use of a protein according to any one of claims 1 to 20 in a bioluminescent assay.

29. A kit comprising a protein according to any one of claims 1 to 20.

30. A kit according to claim 29 which further comprises luciferin.

Fig.1.

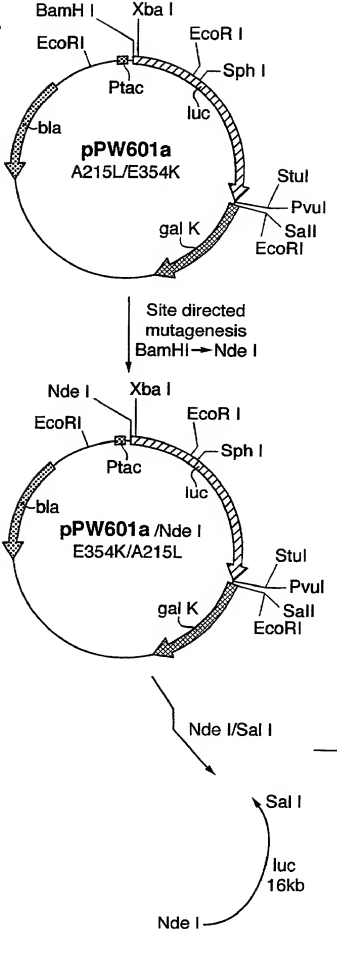

BamH I
Xba I
EcoRI
EcoR I
Sph I
Ptac
luc
bla
pPW601a
A215L/E354K
StuI
PvuI
gal K
SalI
EcoRI
Site directed
mutagenesis
BamHI→Nde I
Nde I
Xba I
EcoRI
EcoR I
Sph I
Ptac
luc
bla
pPW601a /Nde I
E354K/A215L
StuI
PvuI
gal K
SalI
EcoRI
Nde I/Sal I
Sal I
luc
16kb
Nde I

Fig.1 (Cont.)

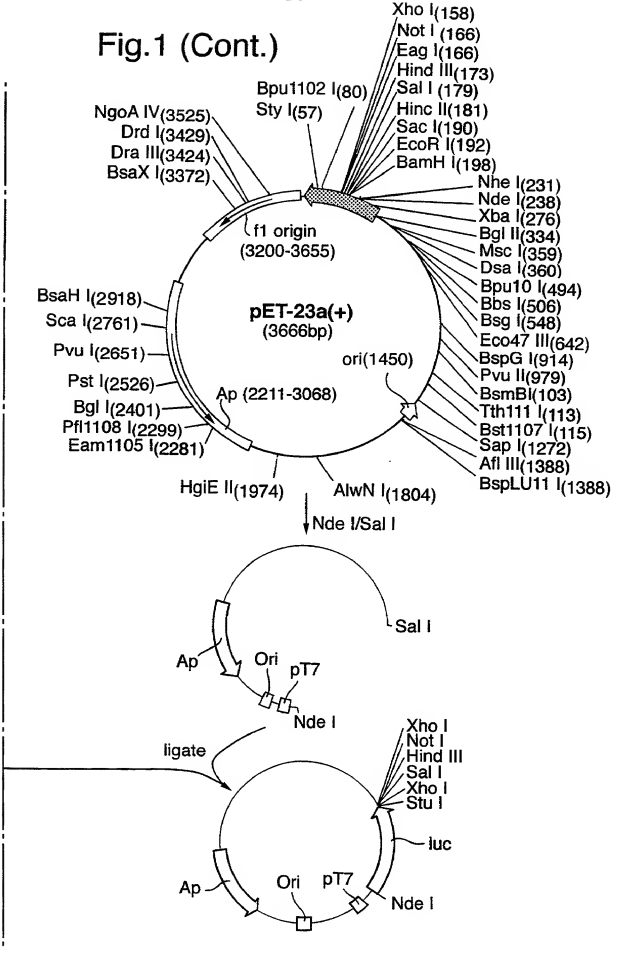

Xho I(158)
Not I (166)
Eag I(166)
Hind III(173)
Sal I (179)
Hinc II(181)
Sac I(190)
EcoR I(192)
BamH I(198)
Nhe I(231)
Nde I(238)
Xba I(276)
Bgl II(334)
Msc I(359)
Dsa I(360)
Bpu10 I(494)
Bbs I(506)
Bsg I(548)
Eco47 III(642)
BspG I(914)
Pvu II(979)
BsmBI(103)
Tth111 I(113)
Bst1107 I(115)
Sap I(1272)
Afl III(1388)
BspLU11 I(1388)
Bpu1102 I(80)
Sty I(57)
NgoA IV(3525)
Drd I(3429)
Dra III(3424)
BsaX I(3372)
f1 origin
(3200-3655)
pET-23a(+)
(3666bp)
ori(1450)
Ap (2211-3068)
BsaH I(2918)
Sca I(2761)
Pvu I(2651)
Pst I(2526)
Bgl I(2401)
Pfl1108 I(2299)
Eam1105 I(2281)
HgiE II(1974)
AlwN I(1804)
Nde I/Sal I
Sal I
Ap
Ori
pT7
Nde I
ligate
Xho I
Not I
Hind III
Sal I
Xho I
Stu I
luc
Ap
Ori
pT7
Nde I

Fig.2.

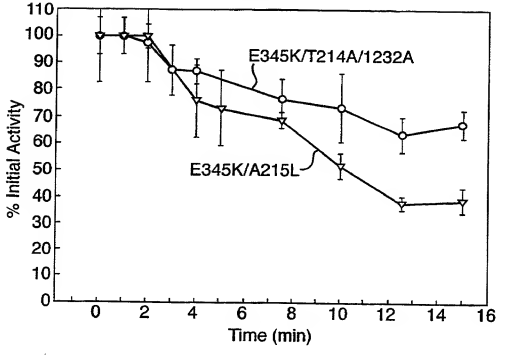

% Initial Activity
Time (min)
E345K/T214A/1232A
E345K/A215L

Fig.4.

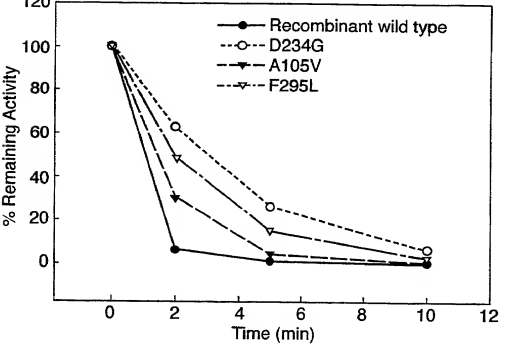

% Remaining Activity
Time (min)
Recombinant wild type
D234G
A105V
F295L

Fig.3a.

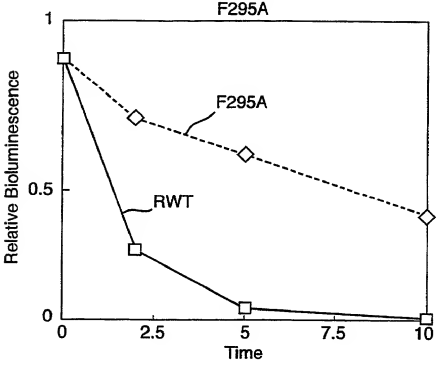

F295A
1
0.5
0
Relative Bioluminescence
F295A
RWT
0
2.5
5
7.5
10
Time

Fig.3b.

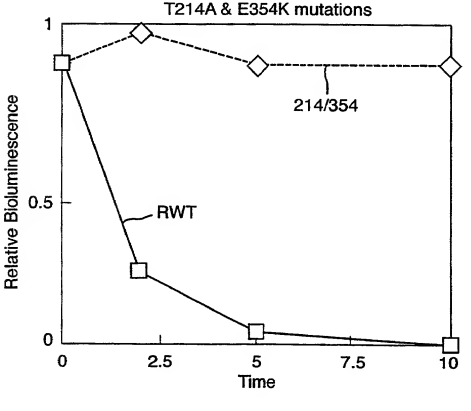

T214A & E354K mutations
1
0.5
0
Relative Bioluminescence
214/354
RWT
0
2.5
5
7.5
10
Time

Fig.3c.

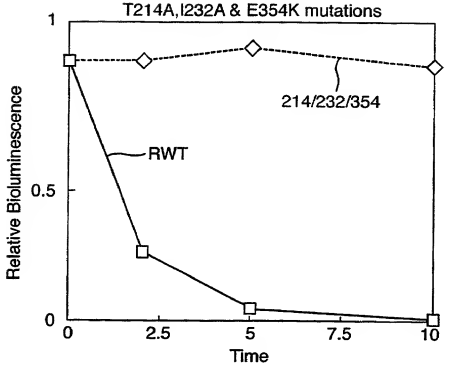

T214A,I232A & E354K mutations
Relative Bioluminescence
1
0.5
0
214/232/354
RWT
0
2.5
5
7.5
10
Time

Fig.3d.

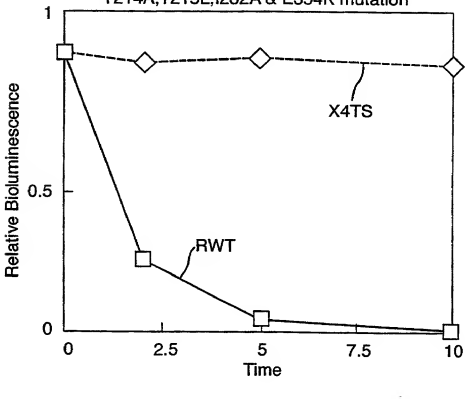

T214A,T215L,I232A & E354K mutation
Relative Bioluminescence
1
0.5
0
X4TS
RWT
0
2.5
5
7.5
10
Time

Fig.3e.

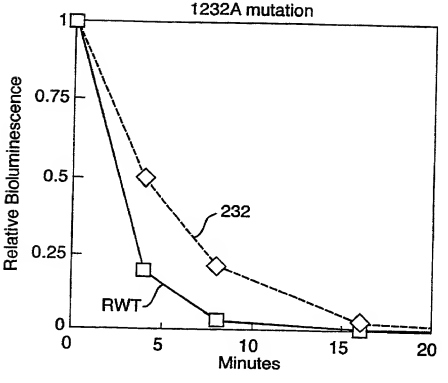

1232A mutation
Relative Bioluminescence
1
0.75
0.5
0.25
0
232
RWT
0
5
10
15
20
Minutes

Fig.3f.

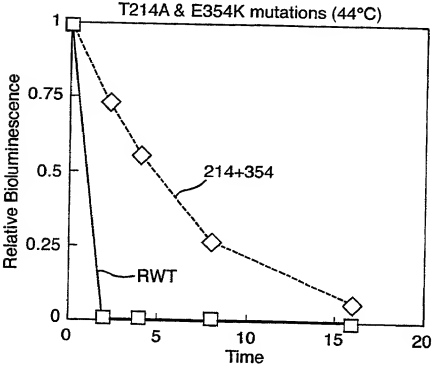

T214A & E354K mutations (44°C)
Relative Bioluminescence
1
0.75
0.5
0.25
0
214+354
RWT
0
5
10
15
20
Time

Fig.3g.

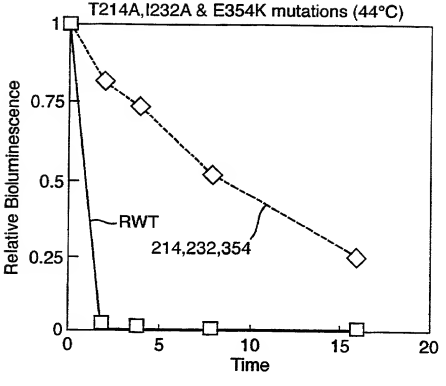
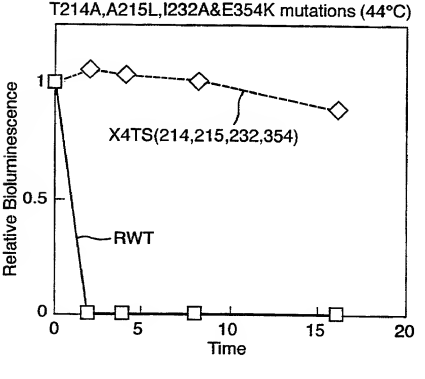

T214A,I232A & E354K mutations (44°C)
Relative Bioluminescence
1
0.75
0.5
0.25
0
RWT
214,232,354
0
5
10
15
20
Time

Fig.3h.


T214A,A215L,I232A&E354K mutations (44°C)
Relative Bioluminescence
1
0.5
0
X4TS(214,215,232,354)
RWT
0
5
10
15
20
Time

# Fig.8.

CGCCGGTGAGCTCCCCGCCGCCG SACI-SENSE / 6371
CGGCGGCGGGGAGCTCACCGGCG SACI-ANTI / 6372
CGAACACTTCTTCATCGTTGACCGCCTTAAGTCTTTAATTAAATACAAAGG AFLII-SENSE / 6373
CCTTTGTATTTAATTAAAGACTTAAGGCGGTCAACTATGAAGAAGTGTTCG AFLII-ANTI / 6374
GAAAGGCCCGGCACCAGCCTATCCTCTAGAGG F14A-SENSE / 6375
CCTCTAGCGGATAGGCTGGTGCCGGGCCTTTC F14A-ANTI / 6376
CCATAAATTTACCGAATTCGTCGACTTCGATCGAGG C-TERM.SEQ/ 6641
GTGTGGAATTGTGAGCGG N-TERM.SEQ / 6651
GAGATACGCCGCGGTTCCTGG L35A-SENSE / 6652
CCAGGAACCGCGGCGTATCTC L35A-SENSE / 6653

CCCTATTTTCATTCCTGGCCAAAAGCACTG F295L-SENSE/ 9048
GAGTGCTTTTGGCCAGGAATGAAAATAGGG F295L-ANTI / 9049
CCGCATAGAgCTCTCTGCGTCAGATTC T214A + A215L-SENSE / 9063
GAATCTGACGCAGAGAGcTCTATGCGG T214A + A215L-ANTI / 9064
GTTGACCGCTTGGGATCCTTAATTAAATAC Insertion of BamHI at G339 / 9077

GTATAGATTTGAAAAAGAGCTG E270K-SENSE / 257
CAGCTCTTTTTCAAATCTATAC E270K-ANTI / 258
GGCTACATACTGGAGACATAGC S420T-SENSE / 629
GCTATGTCTCCAGTATGTAGCC S420T-ANTI / 630
GCAGTTGCGCCCGTGAACGAC A105L-SENSE / 790
GTCGTTCACGGGCGCAACTGC A105L-ANTI /791
CAAATCATTCCGGGTACTGCGATTTTAAG D234G-SENSE / 792
CTTAAAATCGCAGTACCCGGAATGATTTG D234G-ANTI / 793

CCGCATAGAACTCTCTGCGTCAGATTC A215L-SENSE / 7726
GAATCTGACGCAGAGAGTTCTATGCGC A215L-ANTI / 7727
CTGATTACACCCAAGGGGGATG E354K-SENSE / 7792
CATCCCCCTTGGGTGTAATCAG E354K-ANTI / 7793
ccctccgcatagannngcctgcgtcagt T214N-Sense / 8202
actgacgcaggcNNNtctatgcggaaggg T214N-Anti / 82033

GCAATCAAATCGCTCCGGATACTGC I232A-SENSE / 6911
GCAGTATCCGGAGCGATTTGATTGC I232A-ANTI / 6912

CCATTCCATCAAGGTTTTGG H245Q-SENSE / 9128

CCAAAACCTTGATGGAATGG H245Q-ANTI / 9129

**RULE 63 (37 C.F.R. 1.63)**
**DECLARATION AND POWER OF ATTORNEY**
**FOR PATENT APPLICATION**
**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

As below named inventor, I hereby declare that my residence. Post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled.

NOVEL ENZYME.

The specification of which (check applicable box(s)):

[ ] is attached hereto.

[ ] was filed on _____

as U.S. Application Serial No. _____

[ X ] was filed as PCT international application No. PCT/GB99/03538 filed 26 October 1999

and (if applicable to U.S. or PCT Application) was amended on

_____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 C.F.R. 1.56(A). I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

| **Prior Foreign Application(s): Application Number** | **County** | **Day/Month/Year Filed** |
|---|---|---|
| **9823468.5** | **GB** | **28 October 1998** |

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above and below, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56(a) which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

| **Prior U.S. /PCT Applications(s):** | **Day/Month/Year Filed** | **Status:** |
|---|---|---|
| **PCT/GB99/03538** | **26 October 1999** | **Pending** |

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issued thereon. And I hereby appoint **NIXON & VANDERHYE P.C. 8th Floor, 1100 North Glebe Road, Arlington, Virginia 22201-4714 Telephone number (703) 816-4000 to who all communications are to be directed).** And the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C Mitchard, 29009;; Duane M Byers, 33363; Paul J. Henon, 33626; Jeffry H. Nelson, 30481; John R. Lustova, 33149; H. Warren Burnam, Jr., 29366; Thomas E. Byrne, 32205; Mary J Wilson 32955; J Scott Davidson 33489

18

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7.00 **Inventors Signature** _____ Date 22.09.00

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